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Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway

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To cope with life-threatening high osmolarity, yeast activates the high-osmolarity glycerol (HOG) signaling pathway, whose core element is the Hog1 MAP kinase cascade. Activated Hog1 regulates the cell cycle, protein translation, and gene expression. Upstream of the HOG pathway are functionally redundant SLN1 and SHO1 signaling branches. However, neither the osmosensor nor the signal generator of the SHO1 branch has been clearly defined. Here, we show that the mucin-like transmembrane proteins Hkr1 and Msb2 are the potential osmosensors for the SHO1 branch. Hyperactive forms of Hkr1 and Msb2 can activate the HOG pathway only in the presence of Sho1, whereas a hyperactive Sho1 mutant activates the HOG pathway in the absence of both Hkr1 and Msb2, indicating that Hkr1 and Msb2 are the most upstream elements known so far in the SHO1 branch. Hkr1 and Msb2 individually form a complex with Sho1, and, upon high external osmolarity stress, appear to induce Sho1 to generate an intracellular signal. Furthermore, Msb2, but not Hkr1, can also generate an intracellular signal in a Sho1-independent manner.

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Introduction

The budding yeast *Saccharomyces cerevisiae* survive widely fluctuating osmotic conditions in their natural habitat, such as the surface of ripening grapes. To cope with an increased external osmolarity, yeast synthesize, and intracellularly retain the compatible osmolyte glycerol (Gustin *et al*, 1998; Hohmann, 2002). There is also a temporary arrest in cell cycle progression and inhibition of protein translation, during which cells readjust to the changed environment (Bilsland-Marchesan *et al*, 2000; Belli *et al*, 2001; Teige *et al*, 2001; Escot *et al*, 2004). These events are governed by the high-osmolarity glycerol (HOG) signaling pathway, whose core element is the Hog1 MAP kinase (MAPK) cascade. As a result, defects in the HOG pathway cause severe osmosensitivity in cell growth.

The upstream part of the HOG pathway is composed of the functionally redundant, but mechanistically distinct, SLN1 and SHO1 branches (Figure 1A). A signal emanating from either branch converges on a common MAPK kinase (MAPKK), Pbs2, which is the specific activator of the Hog1 MAPK (Brewster *et al*, 1993; Maeda *et al*, 1994, 1995). For yeast to survive on high-osmolarity media, either the SLN1 or the SHO1 branch alone is sufficient.

For each branch, there must be an osmosensor that generates an intracellular signal in response to extracellular osmolarity variations. It is believed that the osmosensor for the SLN1 branch is Sln1, a transmembrane (TM) histidine kinase (Maeda *et al*, 1994). Sln1 detects turgor changes and transmits a signal via the Sln1-Ypd1-Ssk1 phospho-relay system (Posas *et al*, 1996; Reiser *et al*, 2003). Unphosphorylated Ssk1 binds and activates the functionally redundant Ssk2/Ssk22 MAPKK kinases (MAPKKK) that activate the Pbs2 MAPKK (Posas and Saito, 1998).

In contrast, the osmosensor in the SHO1 branch has been elusive. There are three candidates, but none has been convincingly shown to be an osmosensor. The first candidate is the branch's namesake, Sho1, which is, to date, the most upstream known component of the pathway. Sho1 has four TM domains, TM1∼TM4, separated by short loops (Loop-1∼Loop-3) of five to eight amino acids each (Maeda *et al*, 1995) (see Figure 4A for a schematic structure of Sho1). The arrangement of the tightly packed four TM domains is highly conserved across fungi that possess an Sho1 ortholog, suggesting that it may have a more specific function than simple membrane targetting (Krantz *et al*, 2006). Sho1 predominantly localizes to the cytoplasmic membrane at areas of polarized growth, such as the emerging bud and the bud neck (Raitt *et al*, 2000; Reiser *et al*, 2000). The Sho1 C-terminal cytoplasmic region contains an SH3 domain and binds both the Pbs2 MAPKK and the complex of the Ste11 MAPKKK and the Ste50 adaptor protein (Maeda *et al*, 1995; Zarrinpar *et al*, 2004; Tatebayashi *et al*, 2006). Thus, Sho1 serves as an obligatory adaptor between the Ste11 MAPKKK and its substrate Pbs2. It has not, however, been experimentally determined if Sho1 serves an osmosensor function as originally postulated (Maeda *et al*, 1995).

A second candidate for the osmosensor in the SHO1 branch is Msb2. The *MSB2* gene was originally identified as a multicopy suppressor of the budding defect of *cdc24-ts*

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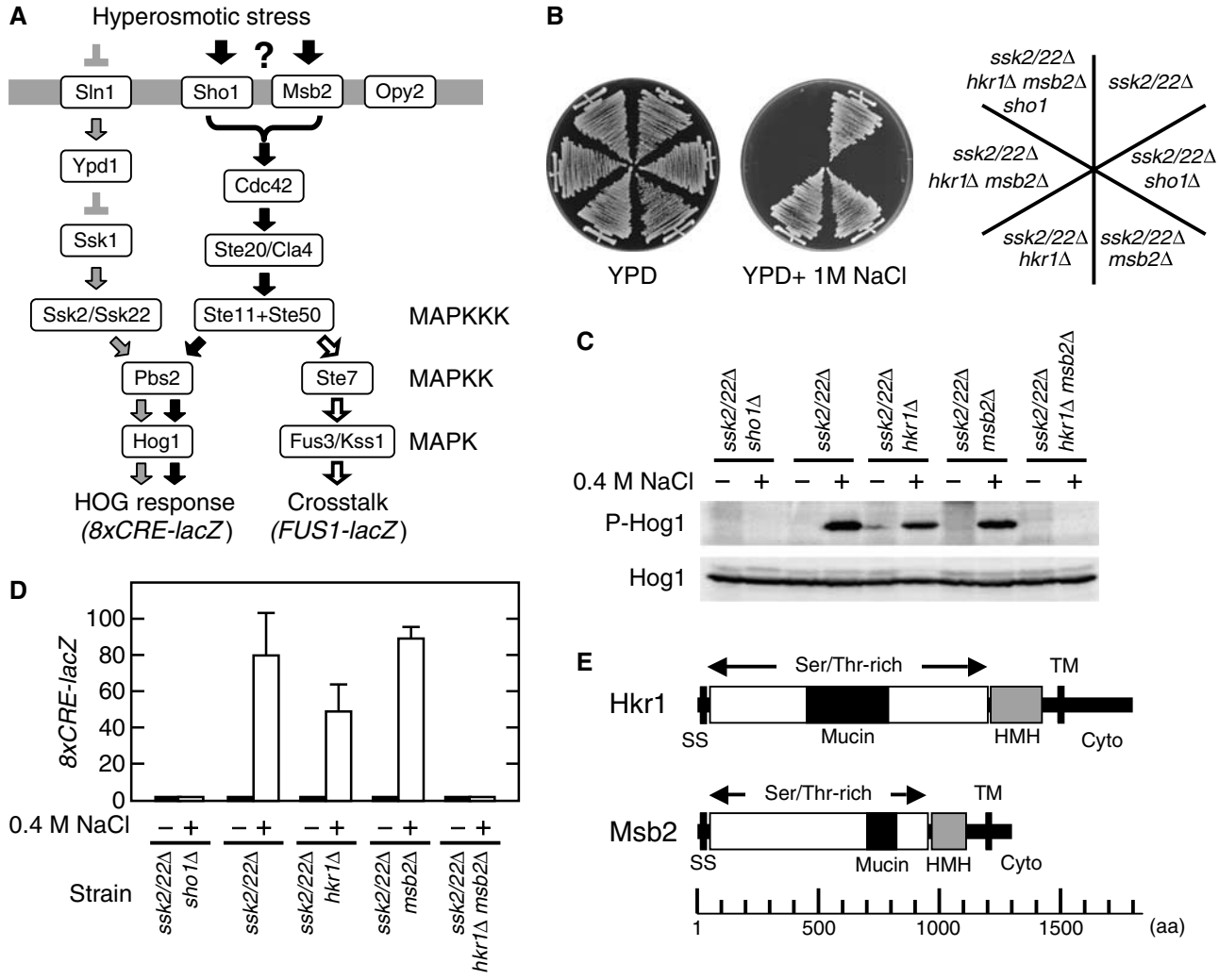

Figure 1 Hkr1 and Msb2 are redundant in the SHO1 branch of the HOG pathway. (**A**) A schematic model of the yeast HOG pathway. The gray horizontal bar represents the plasma membrane. Arrows indicate positive signal flow, whereas perpendicular bars represent negative regulation. The crosstalk pathway is indicated by white arrows. (**B**–**D**) Phenotypes of *hkr1*Δ and *msb2*Δ mutant cells. The following yeast strains were used: TM257 (*ssk2/22*Δ), QG153 (*ssk2/22*Δ *sho1*Δ), KT034 (*ssk2/22*Δ *msb2*Δ), KT060 (*ssk2/22*Δ *hkr1*Δ), KT063 (*ssk2/22*Δ *hkr1*Δ *msb2*Δ), and KT064 (s*sk2/22*Δ *hkr1*Δ *msb2*Δ *sho1*Δ). The complete genotypes of these and other strains used in this work are listed in Supplementary Table I. The *hkr1*Δ *msb2*Δ double mutant is osmosensitive (B), defective in osmostress-induced Hog1 MAPK phosphorylation (C), and defective in osmostress-induced *8xCRE-lacZ* reporter induction (D). Throughout the paper, *8xCRE-lacZ* expression is presented as an average of three or more independent samples, and is expressed in Miller units (Miller, 1972). Where indicated, cells were treated with (+) or without (−) 0.4 M NaCl for 5 min (C) or 30 min (D). (**E**) Schematic models of Hkr1 and Msb2 proteins. Cyto, cytoplasmic domain; HMH, Hkr1-Msb2 Homology domain; SS, signal sequence; ST-rich, serine/threonine-rich; TM, transmembrane segment.

(Bender and Pringle, 1992), and its product is a member of the highly glycosylated mucin family. More recently, it was shown that Msb2 is at the head of the filamentous growth (FG) signal pathway (Cullen *et al*, 2004). In wild-type yeast cells, hyperosmotic stress activates neither the mating pathway nor the FG pathway. However, when osmotic activation of Hog1 is prevented, for example by a *pbs2*Δ or a *hog1*Δ mutation, osmostress induces the mating-specific reporter, *Fus1-lacZ* (Hall *et al*, 1996; O'Rourke and Herskowitz, 1998). This physiologically inappropriate crosstalk, however, also has characteristics of the FG pathway, such as independence from Ste4 and Ste5 and a strong dependence on Ste50 (Cullen *et al*, 2004; O'Rourke and Herskowitz, 1998, 2002). Unlike mating factor, furthermore, osmostress can induce *Fus1-lacZ* even in diploid (*pbs2*Δ/*pbs2*Δ) cells (K. Tatebayashi, unpublished data). Indeed, crosstalk induction of an FG-specific reporter (*FRE-lacZ*) has been observed in *pbs2* mutant cells (Davenport *et al*, 1999). More important, the crosstalk activation of the mating/FG pathways is completely suppressed by a *sho1*Δ *msb2*Δ double mutation, but only partially by *sho1*Δ or *msb2*Δ alone (O'Rourke and Herskowitz, 1998, 2002), suggesting that Sho1 and Msb2 have related roles in the FG and HOG pathways. A physiological role for Msb2 in the HOG pathway, however, has been dismissed, because *msb2*Δ mutants (in a host strain that is defective in the SLN1 branch) are osmoresistant, with robust Hog1 phosphorylation and HOG-dependent gene expression upon osmostress stimulation (O'Rourke and Herskowitz, 2002; Cullen *et al*, 2004).

Finally, a third candidate for the osmosensor in the SHO1 branch is Opy2. Opy2 is a type 1 TM protein, recently shown to have an essential role in the SHO1 branch, as *opy2*Δ *ssk1*Δ double mutants are synthetically osmosensitive (Wu *et al*, 2006). However, there is no evidence that Opy2 participates in an osmosensing process.

Thus, despite much speculation, the identity of the osmosensor in the SHO1 branch has been elusive. Here, we report that two mucin-like TM proteins Hkr1 and Msb2 are the most-upstream components in the SHO1 branch so far identified, and thus are likely candidates for the osmosensors. We also investigate how Sho1 might function with the Hkr1/Msb2 in transmitting the osmostress signal.

Results

Mucin-like transmembrane proteins, Msb2 and Hkr1, are functionally redundant in the SHO1 branch

To search for an osmosensor in the SHO1 branch, we used the following criteria. First, the osmosensor is likely to be a TM protein. Second, null mutants of the sensor will be unable to respond to osmostress. Third, the osmosensor should be the most upstream element in the SHO1 branch. And fourth, certain mutations of osmosensor may alter the sensor's kinetic properties.

According to the first criterion, Msb2 is one of the potential candidates (Figure 1A). It has been dismissed as the osmosensor only because disruption of the *MSB2* gene does not have any appreciable effects on the cell's ability to activate the HOG pathway upon osmostress, or on cellular growth on high-osmolarity media (O'Rourke and Herskowitz, 2002). However, because of the high importance of osmostress signaling for yeast, functional redundancy of key molecules is a recurring feature in the HOG pathway. Thus, if there is a gene that is functionally redundant with *MSB2* in the SHO1 branch, it would mask the essential involvement of Msb2 in the HOG pathway. To test this possibility, we screened for a mutant that is osmosensitive only in an *msb2*Δ background. Note that to focus on the SHO1 branch only, all yeast strains used in this work are of the *ssk2*Δ *ssk22*Δ (hereinafter abbreviated as *ssk2/22*Δ) genetic background, unless stated otherwise. Thus, we mutagenized an *msb2*Δ *ssk2/22*Δ strain with ethyl methanesulfonate, and screened for mutants that were osmosensitive and unable to express the HOG-specific reporter gene *8xCRE-lacZ* (Tatebayashi *et al*, 2006) upon osmotic stress. Into each of the ~350 mutants thus selected, a plasmid encoding the wild-type *MSB2* gene was introduced, and the mutants were screened for those that became both osmoresistant and capable of reporter gene expression. In this manner, we identified three mutants that were both osmosensitive and incapable of expressing the *8xCRE-lacZ* reporter gene, but only in the absence of the *MSB2* gene. To identify the mutant gene responsible for this phenotype, we screened for genomic DNA clones that could complement the osmosensitive defect of the mutants. All three mutants were rescued by genomic DNA clones that contain the *HKR1* gene.

To verify that *hkr1* mutations are responsible for the osmosensitive phenotype of the original mutants, we disrupted the *HKR1* gene in various host cells. As shown in Figure 1B, *hkr1*Δ or *msb2*Δ alone (in the *ssk2/22*Δ background) conferred no osmosensitivity to yeast cells, whereas the *hkr1*Δ *msb2*Δ double-mutant cells were severely osmosensitive. Osmostress-induced phosphorylation of the Hog1 MAPK (which is a measure of Hog1 activation by the Pbs2 MAPKK) was not significantly reduced by *hkr1*Δ or by *msb2*Δ alone, but was completely abolished in the *hkr1*Δ *msb2*Δ double mutant (Figure 1C). Osmostress-induced expression of the HOG-specific reporter, *8xCRE-lacZ*, also followed the same pattern; the *hkr1*Δ *msb2*Δ double mutant was defective in reporter expression, whereas neither *hkr1*Δ nor *msb2*Δ alone reduced the reporter expression significantly (Figure 1D). Thus, Hkr1 and Msb2 serve critical, although redundant, roles in the SHO1 branch.

Hkr1 and Msb2 are single-pass TM proteins of 1802 and 1306 amino acids, respectively (Figure 1E). Their extracellular regions have three notable similarities. First, both have a highly Ser/Thr-rich (STR) domain. Hkr1 residues 51–1200 are 44% Ser/Thr, and Msb2 residues 51–950 are 49% Ser/Thr. Second, within the STR domain, both proteins have tandem Ser/Thr/Pro-rich repeats reminiscent of highly glycosylated mucin proteins, hence termed the mucin repeats (Supplementary Figure S1A and B). The sequences of these repeats, however, are different from each other. Third, immediately following the STR domain, there is a highly homologous region (47% identity; Supplementary Figure S1C) between Hkr1 (residues 1210–1427) and Msb2 (residues 961–1117), hence termed the Hkr1-Msb2 Homology (HMH) domain. There is no significant sequence similarity between the cytoplasmic domains of Hkr1 and Msb2.

Positive- and negative-regulatory domains in Hkr1 and Msb2

To analyze the contribution of each domain of Hkr1 and Msb2 to HOG pathway activation, we constructed various deletions of the *HKR1* and *MSB2* genes (Figure 2A and B). These constructs were individually introduced into an *ssk2/22*Δ *hkr1*Δ *msb2*Δ host strain, and osmotic induction of *8xCRE-lacZ* was measured (Figure 2C and D). The results were essentially identical for the two proteins.

Deletion of the HMH domain (ΔHMH) completely abrogated *8xCRE-lacZ* induction. This is not due to instability or mislocalization of mutant proteins, because expression levels and subcellular localization of Hkr1 ΔHMH-GFP and Msb2 ΔHMH-GFP were not significantly different from those of their full-length parental constructs (Figure 2E–H). The Hkr1 HMH domain contains a central insertion (residues 1296–1357) that has no counterpart in the Msb2 HMH domain (Supplementary Figure S1C). Deletion of the insertion sequence from the Hkr1 HMH domain only moderately reduced Hkr1 activity, whereas deletion of the conserved sequences on either side of the insertion completely abolished Hkr1 activity (Supplementary Figure S2A). Using a series of short deletion mutants of the Msb2 HMH domain, we found that the entire HMH domain, except for the first 18 amino acids, was required for activation of the HOG pathway (Supplementary Figure S2B). We also found that the HMH domains of Hkr1 and Msb2 are functionally interchangeable; replacement of the Msb2 HMH domain with that of Hkr1 did not significantly impair Msb2 function (Supplementary Figure S2C).

Deletion of the entire STR region (ΔSTR) constitutively induced *8xCRE-lacZ* expression, in the absence of any osmostress (Figure 2C and D). A more extensive deletion analysis of the Hkr1 STR region (Supplementary Figure S3) suggested that no specific part of the STR region is required for inhibition, but rather it is the overall length of the STR region that is critical. For example, Hkr1-Δ(50–830) is only moderately hyperactive, whereas Hkr1-Δ(101–1080) is strongly hyperactive. These results indicate, for both Hkr1 and Msb2, that the

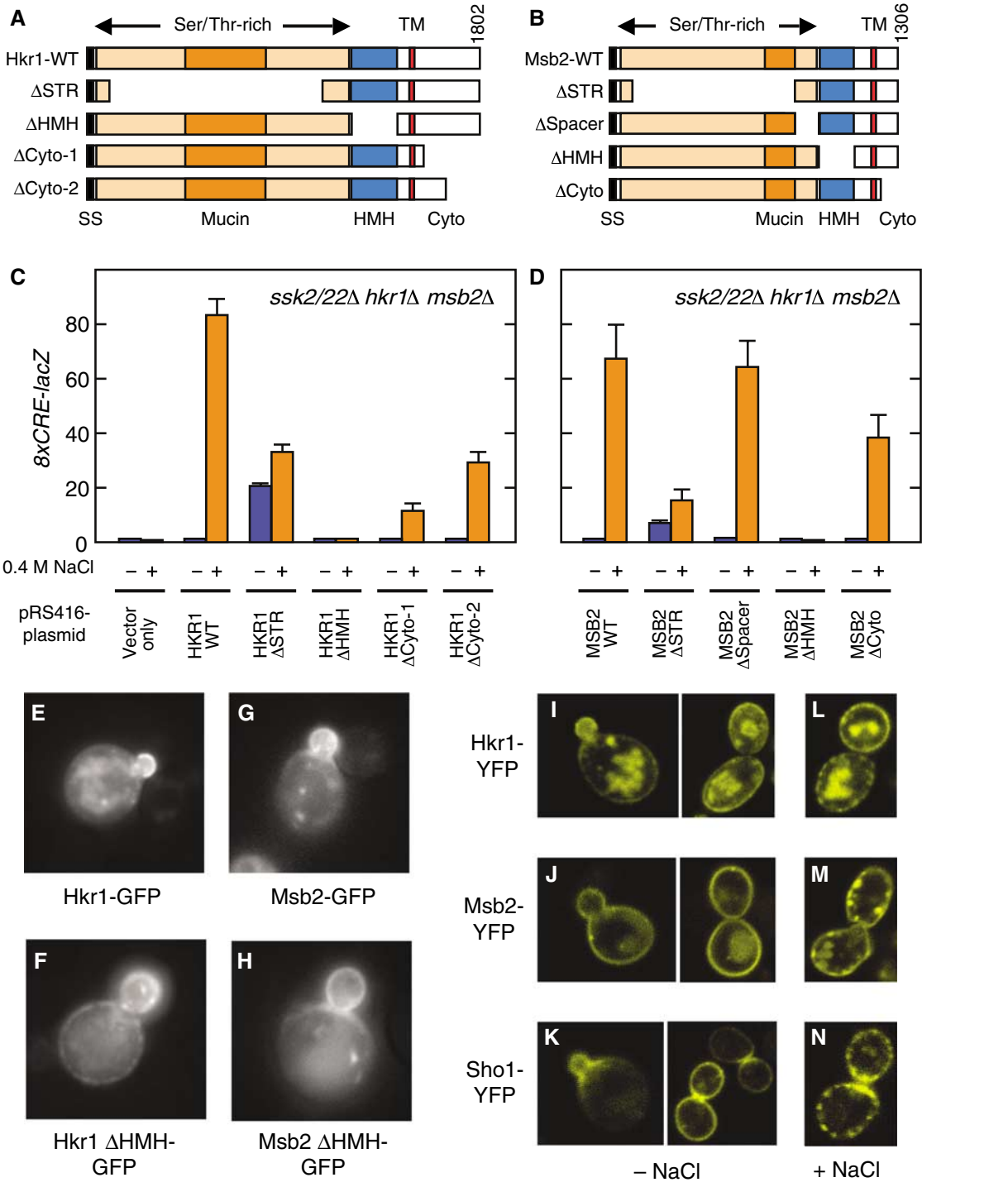

Figure 2 Functional domains and subcellular localization of Hkr1 and Msb2. (**A, B**) Schematic diagrams of the deletion constructs of Hkr1 and Msb2 used in (**C**) and (**D**). Abbreviations are the same as in Figure 1E. WT, wild-type. (C, D) Induction of *8xCRE-lacZ* in KT063 (*ssk2/22*Δ *hkr1*Δ *msb2*Δ) that carries a plasmid encoding either WT or one of the deletion constructs of Hkr1 or Msb2, expressed from their native promoter. (**E–H**) Subcellular localization of Hkr1 and Msb2 in the absence of omsostress. GFP-fusion constructs of full-length Hkr1 (E) and Hkr1 ΔHMH mutant (F) were expressed in KT060 (*ssk2/22*Δ *hkr1*Δ) using pRS426 vector, and full-length Msb2 (G) and Msb2 ΔHMH mutant (H) were expressed in KT034 (*ssk2/22*Δ *msb2*Δ) using pRS416 vector. (**I–N**) Osmostress induces similar relocalization of Hkr1, Msb2, and Sho1. Subcellular localization of Hkr1-YFP (I), Msb2-YFP (J), and Sho1-YFP (K), in the absence of osmostress. Osmostress treatment (0.4 M NaCl for ~10 min) induces a similar punctate redistribution of Hkr1-YFP (L), Msb2-YFP (M), and Sho1-YFP (N). The yeast strain KT064 (*ssk2/22*Δ *sho1*Δ *hkr1*Δ *msb2*Δ) was transformed with the pRS424 vector expressing the indicated fluorescent fusion protein.

STR domain inhibits the signaling function of the essential HMH domain.

Finally, for both proteins, their C-terminal cytoplasmic domain is not essential for HOG pathway activation (Figure 2C and D).

Hkr1 and Msb2 localize to similar membrane sites as Sho1

Sho1 predominantly localizes to the cytoplasmic membrane at areas of polarized growth, such as the emerging bud and the bud neck (Raitt *et al*, 2000; Reiser *et al*, 2000). We thus

determined, by confocal fluorescent microscopy, whether Hkr1 and Msb2 localized in the same subcellular regions as Sho1. The localization of Hkr1 and Msb2 is similar to that of Sho1, although Hkr1 and Msb2 are distributed on the cell surface more uniformly than is Sho1 (Figure 2I–K). Furthermore, osmostress induces a similar punctate redistribution of Hkr1, Msb2, and Sho1 (Figure 2L–N). However, this redistribution occurs in a mutually independent manner—Sho1 redistribution occurs in *hkr1*Δ *msb2*Δ host cells, and Hkr1 and Msb2 redistribution occurs in *sho1*Δ host cells.

Hkr1 and Msb2 are the most-upstream elements in the SHO1 branch known to date

Next, we studied the functional relationship between Hkr1/Msb2 and Sho1 by epistasis analyses. For this purpose, we first analyzed constitutively active Hkr1-ΔSTR and Msb2-ΔSTR constructs. When these proteins were overexpressed in *SHO1*$^+$ cells, using an inducible *GAL1* promoter, the HOG-specific *8xCRE-lacZ* reporter was strongly induced (Figure 3A and C), and so was the activation-associated phosphorylation of the Hog1 MAPK (Figure 3B and D), indicating that the HOG pathway was activated. Overexpression of full-length Hkr1 or Msb2 only very weakly activated the HOG pathway. More important, HOG pathway activation by either Hkr1-ΔSTR or Msb2-ΔSTR was completely inhibited in host cells that are defective in any one of the *SHO1*, *STE20*, *STE50*, *OPY2*, *STE11, PBS2*, and *HOG1* genes (Figure 3E and F and data not shown).

These data place Hkr1 and Msb2 upstream of any other known element in the SHO1 branch of the HOG pathway, although the epistatic relationship between Sho1 and Hkr1/Msb2 needs further analyses (see the next section). This raises the possibility that Hkr1/Msb2 are the osmosensors. If so, appropriate mutations in their genes could conceivably modulate the sensitivity of the cellular response to external osmostress. Indeed, over a range of NaCl concentrations (0.1–0.3 M), Hkr1-Δ(50–830)-expressing cells responded significantly more strongly than Hkr1-WT-expressing cells, whereas their maximal responses at ∼0.4 M NaCl were similar (Figure 3G). In effect, the sensitivity of Hkr1-Δ(50–830) was shifted by ∼50 mM compared to that of wild-type Hkr1.

Constitutively active mutations in the Sho1 extracellular domain activate the HOG pathway in the absence of both Hkr1 and Msb2

The epistasis test in the previous section was incomplete in the sense that it might have only proved that the adaptor function of Sho1 is downstream of Hkr1/Msb2. The Sho1 SH3 domain binds to a Pro-rich motif in Pbs2, and it also interacts with the Ste50 and Ste11 proteins, serving as an adaptor between the Ste50/Ste11 complex and the Pbs2 MAPKK (Maeda *et al*, 1995; Tatebayashi *et al*, 2006). Without this adaptor function, no activation of the HOG pathway occurs. We thus conducted additional epistasis analyses in the reverse direction using a constitutively active mutant that appears to affect a more upstream function of Sho1.

We previously reported several constitutively active Sho1 mutants (e.g., Sho1-R342G) that have mutations in the cytoplasmic domain and have enhanced adaptor function. Those mutants could activate the HOG pathway only in the presence of a constitutively activated Ste11 (Tatebayashi *et al*, 2006). Using a similar screening strategy, we found an additional Sho1 mutant that can activate the HOG pathway, and can do so in the presence of only wild-type Ste11. This mutant, Sho1-P120L, has Pro-120 in the extracellular Loop-3 mutated to Leu (Figure 4A).

Expression of Sho1-P120L induced the HOG pathway reporter *8xCRE-lacZ* (Figure 4B) and phosphorylation of the Hog1 MAPK (Figure 4C) in the wild-type cells, in the absence of any osmostress. To determine whether any other mutation at Pro-120 constitutively activates the HOG pathway better than P120L, we changed Pro-120 to several other nonpolar or neutral amino acids. Of those amino acids tested, P120V, P120C, and P120T could, to varying degrees, induce *8xCRE-lacZ* reporter expression, although none was more effective than the original P120L mutant (Figure 4B and data not shown).

As expected, HOG activation by Sho1-P120L was completely abrogated by deletion of downstream elements in the SHO1 branch, such as *ste20*Δ, *ste50*Δ, and *opy2*Δ in the host strain (Figure 4D), or by the W338F mutation in the Sho1 SH3 domain that blocks interaction with the downstream Pbs2 (Zarrinpar *et al*, 2003) (Figure 4E). In clear contrast, Sho1-P120L can activate the HOG pathway in *hkr1*Δ, *msb2*Δ, or even in *hkr1*Δ *msb2*Δ double-mutant host cells (Figure 4D and E), arguing strongly that Sho1-P120L functions downstream of both Hkr1 and Msb2, but upstream of all other known elements in the SHO1 branch. It should be noted, however, that *hkr1*Δ, and to a lesser extent *msb2*Δ, moderately reduces the reporter expression by Sho1-P120L. Therefore, it is possible that Hkr1 and Msb2, although not essential, might still interact with Sho1-P120L and modulate its activity. Taken together, these results place Hkr1 and Msb2 upstream of all other known elements in the SHO1 branch.

Membrane-anchorage of Ste50 suppresses the opy2 defect

Recently, Wu *et al* (2006) implicated Opy2 in the SHO1 branch. Using the HOG-specific reporter gene *8xCRE-lacZ*, we confirmed their conclusion as shown in Figure 4F. Disruption of *OPY2* in a host that is defective in the SLN1 branch (*opy2*Δ *ssk2/22*Δ) completely abrogated osmotic induction of *8xCRE-lacZ* expression. In contrast, disruption of *OPY2* alone (*opy2*Δ) or together with another gene in the SHO1 pathway (*opy2*Δ *ste11*Δ) did not inhibit reporter induction at all. These results place Opy2 squarely in the SHO1 branch of the HOG pathway. Because Opy2 binds Ste50, it has been proposed that the role of Opy2 might be to recruit Ste50 to the plasma membrane (Wu *et al*, 2006). To test this idea, we used a Ste50 Δ1-Cpr construct in which the C-terminal Cdc42-binding domain of Ste50 (Truckses *et al*, 2006) is replaced by a membrane-targeting C-terminal prenylation site (Cpr) of Ras2 (Tatebayashi *et al*, 2006). The *opy2*Δ *ssk2/22*Δ cells could activate the HOG pathway efficiently by osmostress if Ste50 Δ1-Cpr was expressed in the cells (Figure 4G). In clear contrast, the presence of Ste50 Δ1-Cpr did not ameliorate the signaling defect of *hkr1*Δ *msb2*Δ *ssk2/22*Δ mutant cells (data not shown). Thus, it is likely that the essential role of Opy2 is to recruit the Ste50 adaptor protein to the plasma membrane, rather than to play a role in osmosensing. That the site of Opy2 action is downstream of both Hkr1/Msb2 and the TM function of Sho1, as deduced from

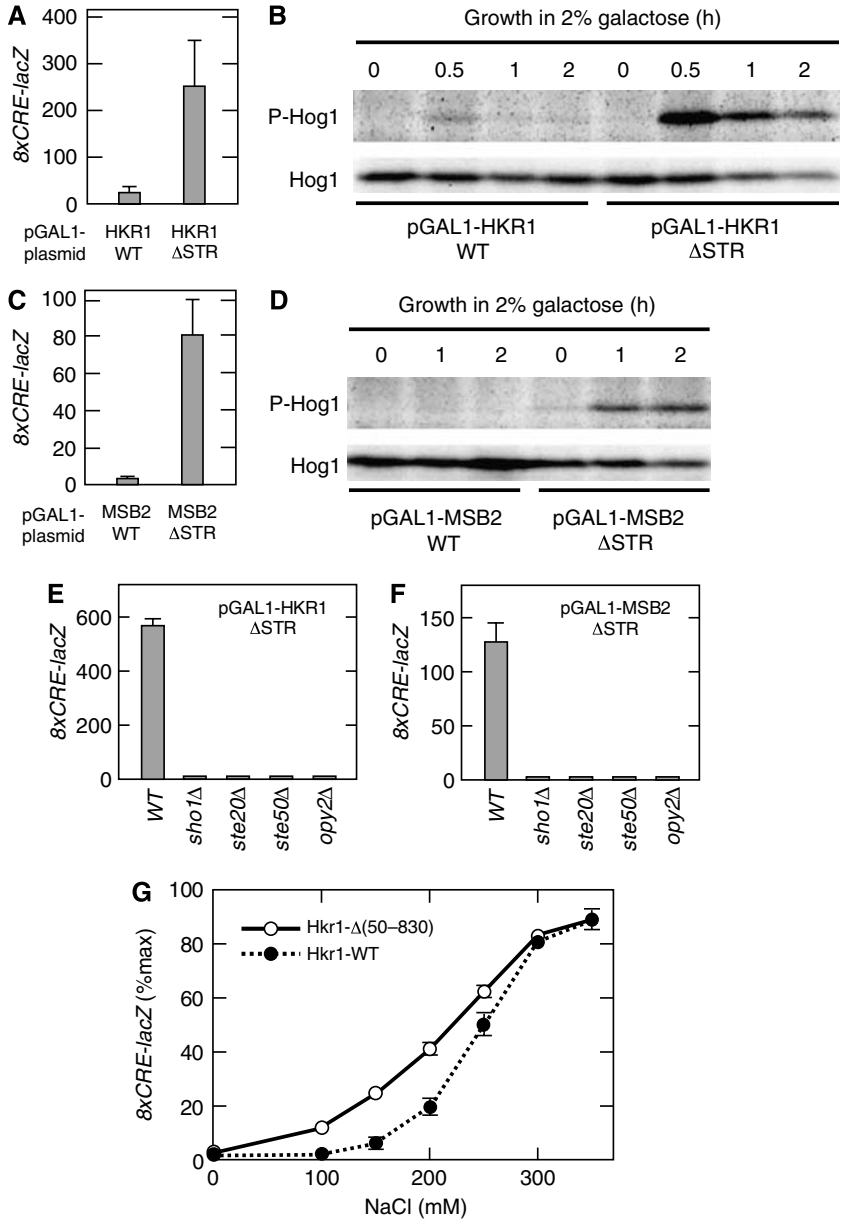

Figure 3 Constitutively active mutants of Hkr1 and Msb2 indicate that they are upstream of any other known element in the SHO1 branch. (**A–D**) Constitutively active *HKR1ΔSTR* or *MBS2ΔSTR*, placed in a single-copy plasmid with the *GAL1* promoter, was induced by galactose. TM257 (*ssk2/22*Δ) was used. Expression of *HKR1ΔSTR* induces the HOG pathway reporter gene *8xCRE-lacZ* (A) and phosphorylation of Hog1 (B). Expression of *MSB2ΔSTR* also induces the HOG pathway reporter (C) and phosphorylation of the Hog1 MAPK (D). (**E, F**) Activation of the HOG pathway by constitutively active Hkr1 or Msb2 is dependent on Sho1, Ste20, Ste50, and Opy2. Induction of *8xCRE-lacZ* by expression of *HKR1ΔSTR* (E) or *MSB2ΔSTR* (F) was assayed in mutant cells of the indicated genotypes. Yeast strains used were TM257 (WT), KT064 (*sho1*Δ), KT032 (*ste20*Δ), FP67 (*ste50*Δ), and KY477 (*opy2*Δ). (**G**) Altered sensitivity to osmostress of an Hkr1 STR domain deletion mutant. The yeast strains KT034 (*ssk2/22*Δ *msb2*Δ *HKR1*$^{+}$) and TA039 (*ssk2/22*Δ *msb2*Δ *hkr1−Δ(50–830)*) carrying an *8xCRE-lacZ* reporter plasmid were stimulated with the indicated concentration of NaCl for 30 min. *8xCRE-lacZ* expression was normalized as the percentage of the maximum expression that occurs at 0.4 M for both strains.

the data in Figures 3E, F and 4D, is consistent with this interpretation.

Msb2 can activate the HOG pathway by two different mechanisms

The finding that a Sho1 mutant in the extracellular loop (P120L) is hyperactive implies that the extracellular loops, and presumably the TM domains, have an active role in osmostress signaling. The properties of Myr-Sho1, however, appear to contradict this interpretation. Myr-Sho1 is a Sho1 derivative in which the extracellular loops and the four TM segments are entirely replaced by the membrane-targeting myristoylation signal of Gpa1 (see Figure 5A). Myr-Sho1 can functionally complement *sho1*Δ, as assayed by cell growth on high-osmolarity media, and by osmotic activation of the Hog1 MAPK (Raitt *et al*, 2000). These results were confirmed using the more quantitative *8xCRE-lacZ* reporter assay. Thus, whereas *ssk2/22*Δ *sho1*Δ mutant cells did not induce the reporter gene upon osmostress stimulation, the same cells expressing Myr-Sho1 responded at about one-third of the

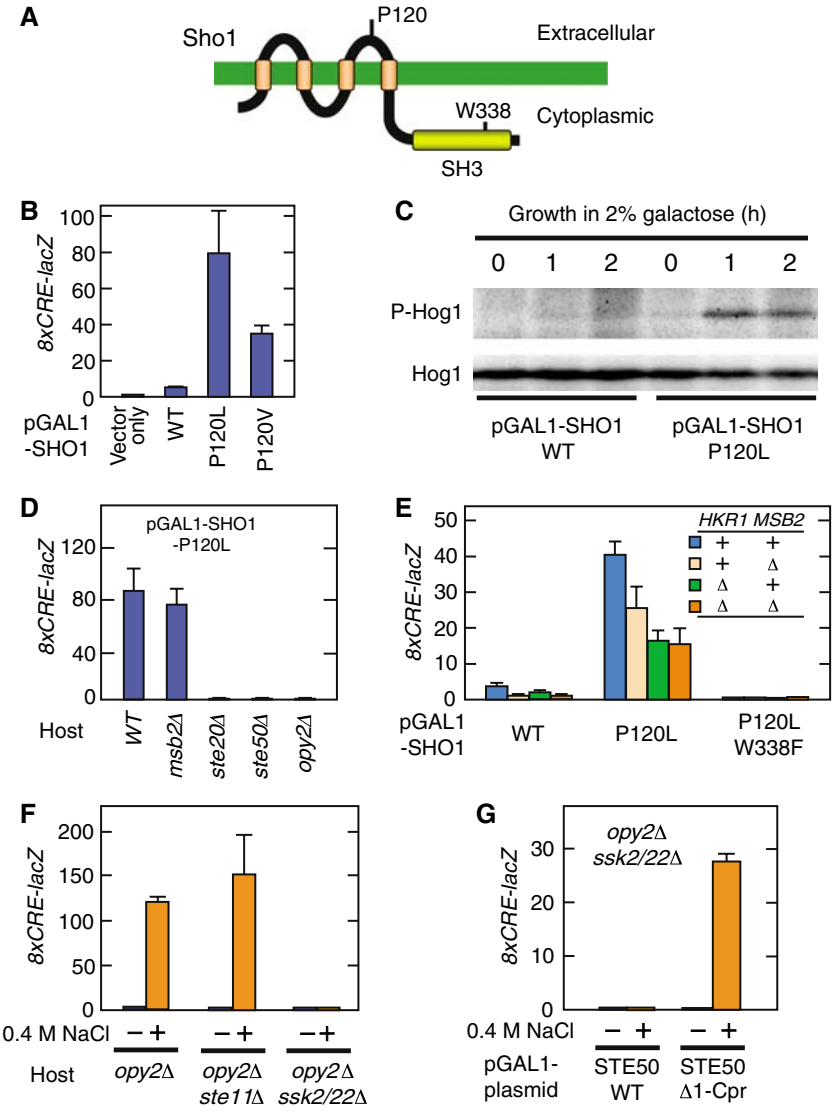

Figure 4 Sho1 functions downstream of Hkr1/Msb2. (**A**) A schematic model of Sho1. The horizontal bar represents the plasma membrane. Approximate positions of Pro-120 (P120) and Trp-338 (W338) are indicated. (**B–C**) Expression of constitutively active Sho1-P120L induces the HOG pathway reporter gene *8xCRE-lacZ* (B) and phosphorylation of Hog1 (C). (**D**) Activation of the HOG pathway by Sho1-P120L is dependent on Ste20, Ste50, and Opy2. Constitutively active *SHO1-P120L*, placed in a single-copy plasmid with the *GAL1* promoter, was induced by galactose for 2 h, following which cell extracts were prepared for reporter assays. Yeast strains used were TM257 (wild-type (WT)), KT034 (*msb2*Δ), KT032 (*ste20*Δ), FP67 (*ste50*Δ), and KY477 (*opy2*Δ). (**E**) Sho1-P120L can activate the HOG pathway in the absence of both Hkr1 and Msb2. WT, or the indicated *SHO1* mutant, was expressed from the *GAL1* promoter for 2 h before reporter activity was measured (without osmostress stimulation). Yeast strains used were QG153 (*sho1*Δ), KT053 (*sho1*Δ *msb2*Δ), KT061 (*sho1*Δ *hkr1*Δ), and KT064 (*sho1*Δ *hkr1*Δ *msb2*Δ). (**F**) Opy2 is essential in the SHO1 branch signaling. Induction of the *8xCRE-lacZ* reporter gene by osmostress was assayed in host cells of the indicated genotypes. Cells were treated with (+) or without (−) 0.4 M NaCl for 30 min before reporter assay. Yeast strains used were KY475 (*opy2*Δ), KY476 (*opy2*Δ *ste11*Δ), and KY477 (*opy2*Δ *ssk2/22*Δ). (**G**) Membrane targeting of Ste50. WT *STE50* or *STE50-*Δ*1-Cpr* (Tatebayashi *et al*, 2006) was expressed in KY477 from the *GAL1* promoter for 1.5 h, and cells were treated with (+) or without (−) 0.4 M NaCl for 30 min before *8xCRE-lacZ* reporter assay.

wild-type Sho1 level (Figure 5B). MyrAS-Sho1, with a defective myristoylation site, did not support the reporter expression at all. Thus, these data would suggest that the sole function of the Sho1 TM segments is to anchor the essential cytoplasmic domain to the plasma membrane (Raitt *et al*, 2000; Tatebayashi *et al*, 2006).

If this was the case, however, Myr-Sho1 should also support activation of the HOG pathway by constitutively active Hkr1-ΔSTR or Msb2-ΔSTR. As shown in Figure 5C, however, neither Hkr1-ΔSTR nor Msb2-ΔSTR could induce the HOG-pathway reporter in Myr-Sho1 mutant cells. To find a clue to this puzzle, we searched for a gene that is required for HOG activation in *Myr-Sho1* mutant cells, but not required in *SHO1*$^+$ cells. To our surprise, *MSB2* itself satisfied this criterion. As seen in Figure 5D, HOG reporter expression by osmostress in *Myr-Sho1* cells (which is also *ssk2/22*Δ to inactivate the SLN1 branch) was completely abrogated by the *msb2*Δ mutation. Disruption of the *HKR1* gene had no effect. Consistent with the reporter expression, *Myr-Sho1 msb2*Δ cells are severely osmosensitive, whereas *Myr-Sho1* and *Myr-Sho1 hkr1*Δ cells are osmoresistant (Figure 5E). The role of Msb2 in *Myr-Sho1* cells is not identical to that in *SHO1*$^+$ cell. In *SHO1*$^+$ host cells, the cytoplasmic domain of Msb2 is not essential for HOG activation by osmostress (see

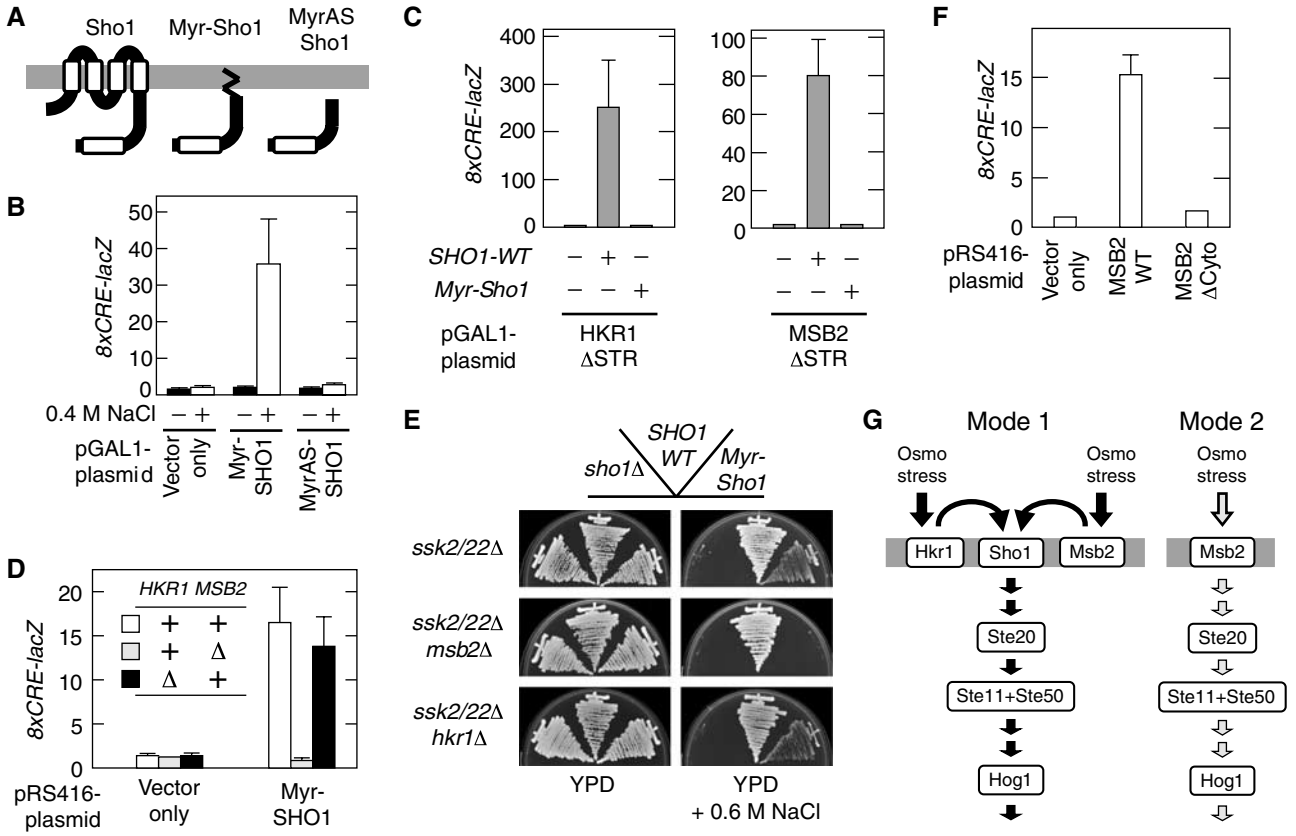

Figure 5 Msb2 can activate the HOG pathway by two different mechanisms. (**A**) Schematic diagrams of wild-type (WT) Sho1 and the Myr-Sho1 fusion protein. The gray horizontal bar represents the plasma membrane. MyrAS-Sho1 is a derivative of Myr-Sho1 with a myristoylation-defective mutation (Raitt *et al*, 2000). (**B**) Induction of the HOG pathway by osmostress in Myr-Sho1 mutant cells. Myr-Sho1 or MyrAS-Sho1 was expressed from the *GAL1* promoter for 1.5 h, using the yeast strain QG153 (*ssk2/22*Δ *sho1*Δ). Cells were then treated with (+) or without (−) 0.4 M NaCl for 30 min before reporter assay. (**C**) Activation of the HOG pathway by constitutively active Hkr1-ΔSTR or Msb2-ΔSTR is dependent on the extracellular/TM segment of Sho1. *HKR1-ΔSTR* or *MSB2-ΔSTR* were expressed from the *GAL1* promoter for 2 h before reporter assay, using the yeast strains KT064 (*ssk2/22*Δ *sho1*Δ *hkr1*Δ *msb2*Δ; left panel) or KT053 (*ssk2/22*Δ *sho1*Δ *msb2*Δ; right panel) carrying a single-copy plasmid encoding either WT *SHO1*, *Myr-SHO1*, or empty vector, as indicated. (**D**) Induction of the HOG-specific reporter *8xCRE-lacZ* in *Myr-SHO1* mutant cells. QG153, KT053, or KT061 (*ssk2/22*Δ *sho1*Δ *hkr1*Δ) carrying the *8xCRE-lacZ* reporter plasmid and either pRS416-Myr-Sho1 (expressed from the *SHO1* promoter) or the empty vector (pRS416) was treated with 0.4 M NaCl for 30 min before reporter assay. (**E**) Osmosensitivity of Myr-Sho1 mutant cells. The *SHO1* genotypes of the strains are shown above, and other relevant genotypes are on the left. Yeast strains used were KT079 (*ssk2/22*Δ *sho1*Δ), KT053, and KT088 (*ssk2/22*Δ *sho1*Δ *hkr1*Δ). (**F**) In Myr-Sho1 cells, the C-terminal cytoplasmic tail of Msb2 is necessary to activate the HOG pathway by osmostress. The yeast strain KT053 carrying pRS414-Myr-Sho1 (expressed from the *SHO1* promoter) was transformed with another single-copy plasmid encoding either WT Msb2 or Msb2-ΔCyto. *8xCRE-lacZ* reporter activity was measured after cells were treated with 0.4 M NaCl for 30 min. (**G**) A schematic model of the two distinct mechanisms of activation in the SHO1 branch. Either Hkr1 or Msb2 can activate the SHO1 branch via Sho1 (mode 1). Msb2 (but not Hkr1) can also activate the SHO1 branch without the participation of the TM segments of Sho1 (mode 2). Because the cytoplasmic domain of Sho1 contains the essential SH3 domain, Sho1 is actually required for both modes.

Figure 2D). In clear contrast, the Msb2 cytoplasmic domain is absolutely required in *Myr-Sho1* host cells (Figure 5F).

Thus, we conclude that Msb2 activates the SHO1 branch by two separate mechanisms (Figure 5G). One mechanism (mode 1) depends on the Sho1 TM domains, but does not require the Msb2 cytoplasmic domain (as demonstrated in Figure 2D). The second mechanism (mode 2) does not require the Sho1 TM domains, but does involve the Msb2 cytoplasmic domain. Hkr1 activates the HOG pathway only by the mode 1 mechanism.

To define the region in the Msb2 cytoplasmic tail necessary for the mode 2 mechanism, a series of 10-aa deletion mutants were generated between residue 1216 and the C terminus (residue 1306). None was defective in HOG activation in the *ssk2/22*Δ *SHO1*$^{+}$ *hkr1*Δ host cells (Supplementary Figure S4B, upper panel), whereas the two most C-terminal deletions, Δ(1286–1295) and Δ(1296–1306) were completely defective in the *ssk2/22*Δ *Myr-SHO1* host cells (Supplementary Figure S4B, lower panel). The essential region was further mapped by replacing three-amino-acid blocks between residue 1289 and the C terminus by Ala-Ala-Ala (AAA). All AAA-substitution mutants, with the exception of the very C-terminal (1304–1306)AAA, were defective in the *ssk2/22*Δ *Myr-SHO1* host cells (Supplementary Figure S4C), indicating that the residues between 1289 and 1303 (underlined in Supplementary Figure S4A) are essential for Msb2 to activate the HOG pathway by the mode 2 mechanism. Sequence comparison of several yeast species revealed that the Msb2 cytoplasmic domain is relatively poorly conserved, with the exception of the C-terminal residues that are needed for the Msb2 mode 2 function (Supplementary Figure S4A). It is likely that this conserved region is involved in cytoplasmic signal generation by Msb2, but its molecular mechanism is not understood.

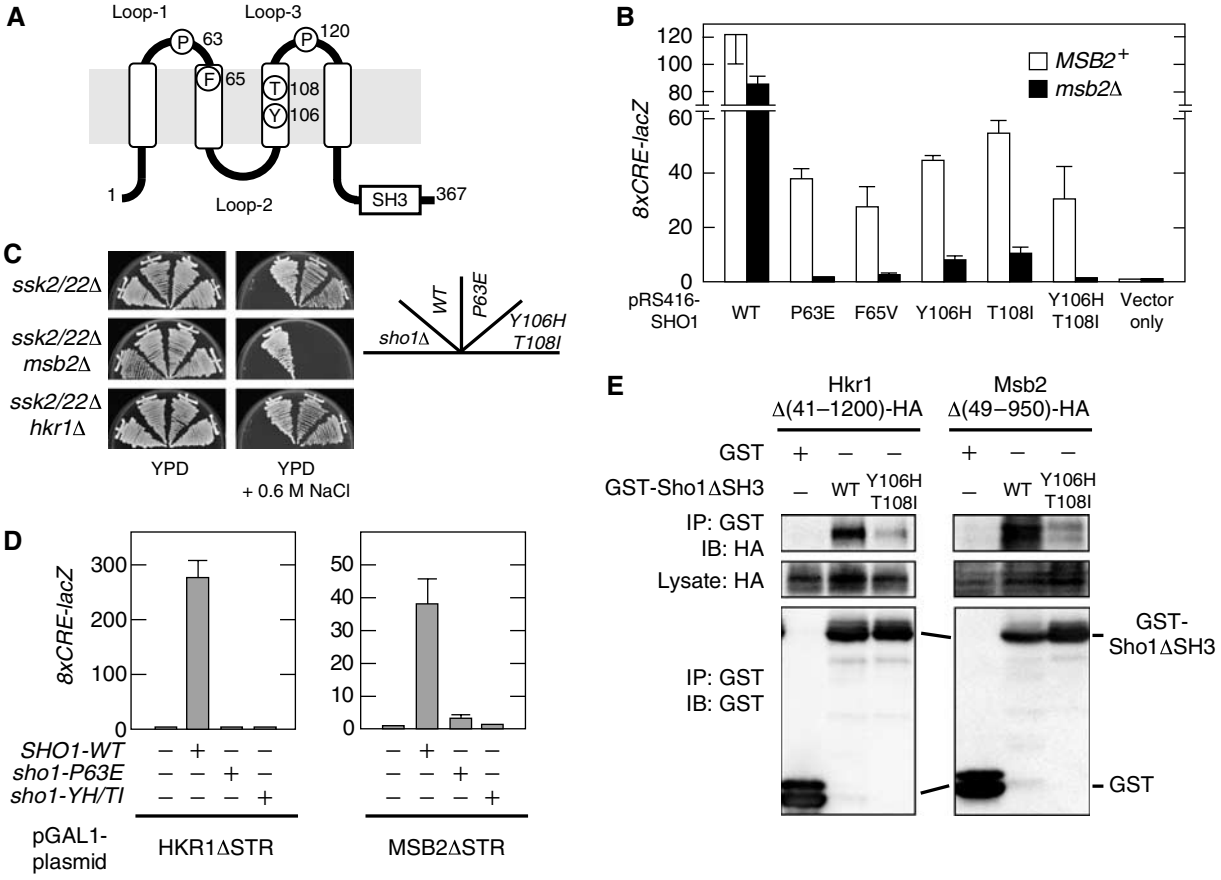

Figure 6 Sho1 mutants that cannot be activated by Hkr1/Msb2. (**A**) A schematic model of the Sho1 protein. Amino-acid residues whose mutation results in constitutively active (P120) or defective (all others) signaling are shown. Not drawn to scale. (**B**) Induction of the HOG pathway by osmostress in various *sho1* mutant cells. Wild-type (WT) *SHO1* or the indicated *sho1* mutant was expressed from the *SHO1*'s own promoter in the yeast strain KT053 (*ssk2/22*Δ *sho1*Δ *msb2*Δ). Cells also carry an *8xCRE-lacZ* reporter plasmid and either an expression plasmid for *MSB2* (*MSB2*$^+$) or the empty vector (*msb2*Δ). Cells were treated with (+) or without (−) 0.4 M NaCl for 30 min before *8xCRE-lacZ* reporter assay. (**C**) Osmosensitivity of *sho1* mutant cells. The *SHO1* genotypes of the strains are shown above, and other relevant genotypes are on the left. Yeast strains used were KT079 (*ssk2/22*Δ *sho1*Δ), KT053, and KT088 (*ssk2/22*Δ *sho1*Δ *hkr1*Δ). (**D**) Activation of the HOG pathway by constitutively active Hkr1-ΔSTRor Msb2-ΔSTR in *sho1* mutants of the extracellular/TM segment. *HKR1-ΔSTR* or *MSB2-ΔSTR* were expressed from the *GAL1* promoter for 2 h before reporter assay, using the yeast strains KT064 (*ssk2/22*Δ *sho1*Δ *hkr1*Δ *msb2*Δ) carrying a single-copy plasmid encoding either WT *SHO1*, *sho1-P63E, sho1-Y106H/T108I* (*YH/TI*), or empty vector, as indicated. (**E**) Sho1 interacts with Hkr1 and Msb2. GST-Sho1ΔSH3 or its Y106H/T108I derivative (or control GST) and HA-tagged Hkr1-Δ(41–1200) or Msb2-Δ(49–950) were expressed from the *GAL1* promoter for 3 h in the yeast strain KT075 (*sho1*Δ *hkr1*Δ *msb2*Δ *pbs2*Δ). GST-Sho1 was precipitated with Glutathione-Sepharose beads, and co-precipitated HA-tagged Hkr1 or Msb2 was detected by immunoblotting.

Interaction between Hkr1/Msb2 and Sho1 is essential for HOG activation by the mode 1 mechanism

The presence of the mode 2 mechanism made the analysis of the immediate subject of this study, namely mode 1, more complex. Ironically, however, it also helped to reveal the mode 1-specific function of Sho1. In other words, it allowed us to isolate Sho1 mutants that are defective only in the signaling between Hkr1/Msb2 and Sho1 (i.e., the mode 1 mechanism), but retains the intact cytoplasmic adaptor function that is needed for both the mode 1 and mode 2 mechanisms. Thus, we screened for *sho1* missense mutants that do not support the HOG signaling in *msb2*Δ *HKR1*$^+$ host cells, but do support the signaling in *MSB2*$^+$ *hkr1*Δ host cells.

Several mutants of such a phenotype were isolated, including P63E, F65V, Y106H, and T108I. Pro-63 is in the extracellular Loop-1, Phe-65 is in TM2, and Tyr-106 and Thr-108 are in TM3 (Figure 6A). Each of these mutant supports osmotic induction of the *8xCRE-lacZ* reporter as long as wild-type Msb2 is present (Figure 6B). In *msb2*Δ host cells, however, those mutant could only poorly induce the reporter. Although Y106H and T108I, individually, have relatively high reporter induction levels in *msb2*Δ hosts, the Y106H T108I double mutant is severely defective. Consistent with the reporter expression pattern, both *sho1-P63E* and *sho1-Y106H T108I* mutants are osmosensitive in an *msb2*Δ background, but not in an *hkr1*Δ background (Figure 6C). More important, neither *sho1-P63E* nor *sho1-Y106H T108I* could support HOG reporter expression induced by constitutively active Hkr1-ΔSTR or Msb2-ΔSTR (Figure 6D).

Previously, Cullen *et al* (2004) has shown that full-length Msb2 binds Sho1. We confirmed the association between Msb2 and Sho1, and demonstrated that Hkr1 too bound Sho1, using constitutively active Msb2 and Hkr1 mutant constructs (Figure 6E). Sho1-P63E could bind Hkr1 and Msb2 as efficiently as the wild-type Sho1 protein (data not shown). However, we found that Sho1-Y106H T108I has lost most of its capacity to bind Hkr1 and Msb2 (Figure 6E). Although the HMH domain is essential, ΔHMH deletion

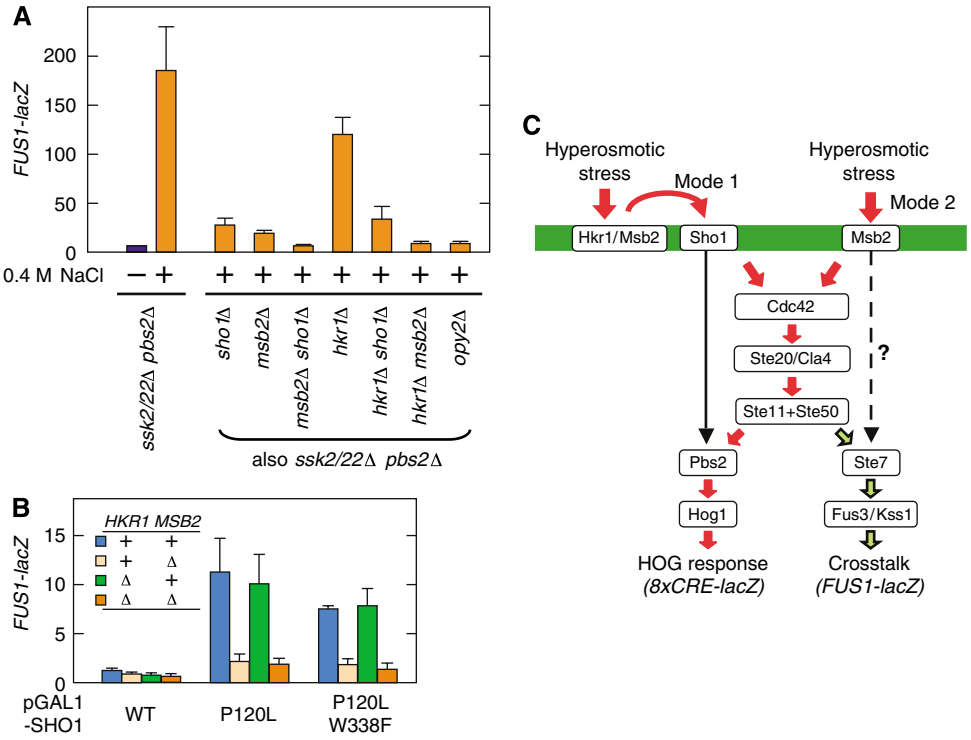

Figure 7 Crosstalk activation of the mating pathway by osmostress. (**A**) Induction of the mating pathway reporter *FUS1-lacZ* by osmostress. Matching strains of the indicated genotypes were treated with (+) or without (−) 0.4 M NaCl for 3.5 h before *FUS1-lacZ* reporter assay. (**B**) Crosstalk activation of the mating pathway by constitutively-active Sho1-P120L. Wild-type (WT) or the indicated *SHO1* mutant was expressed from the *GAL1* promoter for 2 h before *FUS1-lacZ* reporter activity was measured (without osmostress stimulation). Yeast strains used were HY001 (*pbs2*Δ *sho1*Δ), KT052 (*pbs2*Δ *sho1*Δ *msb2*Δ), KT069 (*pbs2*Δ *sho1*Δ *hkr1*Δ), and KT065 (*pbs2*Δ *sho1*Δ *hkr1*Δ *msb2*Δ). (**C**) A revised schematic model of the yeast HOG and crosstalk pathways. The SLN1 branch is omitted.

mutants of Msb2 and Hkr1 could bind Sho1 (data not shown). Thus, we conclude that Hkr1 and Sho1 (and Msb2 and Sho1) interact mainly through their TM domains, and disruption of such interaction by Sho1-Y106H T108I inhibits signaling between Hkr1/Msb2 and Sho1, in the mode 1 activation mechanism.

Roles of Msb2 in the crosstalk activation of the mating pathway by osmostress

Finally, we investigated the roles of Hkr1 and Msb2 in crosstalk activation of the mating pathway. The mating pathways are inappropriately activated by osmostress, when the Ste11 MAPKKK is activated via the SHO1 branch, whereas activation of the Hog1 MAPK is inhibited, for example, by *pbs2*Δ.

In Figure 7A, we examined the crosstalk in various mutant strains by measuring osmotic induction of the *FUS1-lacZ* reporter. All mutants are in a *pbs2*Δ background to prevent Hog1 activation. As previously reported (O'Rourke and Herskowitz, 2002), either *sho1*Δ or *msb2*Δ alone substantially reduced the crosstalk signaling, and *sho1*Δ *msb2*Δ double mutation completely abolished the inappropriate crosstalk. These results can be interpreted as indicating that *sho1*Δ mutant can activate the SHO1 branch by mode 2, whereas *msb2*Δ mutant can activate the SHO1 branch by mode 1. Examination of additional mutants corroborates this view. The *hkr1*Δ mutation only moderately prevents the crosstalk, presumably because Msb2 can activate the SHO1 branch by both mode 1 and 2. The *hkr1*Δ *sho1*Δ double mutation does not prevent the crosstalk any more than *sho1*Δ alone, because only mode 2 is functional in either case. In contrast, *hkr1*Δ *msb2*Δ double mutation completely prevented the crosstalk, as both modes are defective. Thus, these results are, at least qualitatively, consistent with the two-modes mechanism of SHO1 branch activation.

There is, however, a quantitative discrepancy between HOG pathway activation and crosstalk activation. Because osmostress activates the HOG pathway in *msb2*Δ mutant to a similar degree as in *hkr1*Δ mutant (Figure 1D), we can expect that the Ste11 MAPKKK is also activated to similar extents in these mutants. Nevertheless, *msb2*Δ prevents crosstalk much more strongly than *hkr1*Δ, suggesting that Msb2 might have an additional role in crosstalk.

To test this possibility, we examined the crosstalk activation by constitutively active Sho1-P120L. As we have shown in Figure 4D and E, Sho1-P120L can efficiently activate the HOG pathway in *msb2*Δ cells, indicating that Ste11 (the last common element between the HOG pathway and the crosstalk pathway) is efficiently activated in the absence of Msb2. In the cells of the same background (plus *pbs2*Δ), however, crosstalk activation of *Fus1-lacZ* by Sho1-P120L was strongly suppressed by *msb2*Δ (but not by *hkr1*Δ) (Figure 7B). We also examined the effect of the Sho1-W338F mutation that disrupts the proline-motif-binding capacity of the SH3 domain. Activation of the HOG pathway by Sho1-P120L was completely suppressed by W338F as shown in Figure 4E. In clear contrast, the crosstalk activation is largely indifferent to the W338F mutation, indicating that activation of Ste11 is not inhibited by W338F (Figure 7B). Thus, Sho1-W338F inhibits

HOG pathway activation, not because it cannot activate Ste11, but more likely because it cannot tether the activated Ste11 to Pbs2 (Tatebayashi *et al*, 2006).

A revised model of the HOG and crosstalk pathways emerged from this study is schematically shown in Figure 7C. Activation of Ste11 by osmostress can proceed either via mode 1 (Hkr1/Msb2 and Sho1) or via mode 2 (Msb2). Activation beyond Ste11, however, requires additional involvement of Sho1 (for the HOG pathway) or Msb2 (for the crosstalk), perhaps serving scaffold-like functions.

Discussion

We report in this paper three novel findings that change the conceptual framework of the signaling mechanism in the SHO1 branch of the HOG osmoregulatory pathway. The first is the identification of Hkr1 and Msb2 as potential osmosensors in the SHO1 branch. These proteins satisfy all of the four criteria we put forth at the beginning of the Results section: they are TM proteins; when both of their genes are disrupted, mutant cells are incapable of activating the HOG pathway and are severely osmosensitive; they function upstream of all other known elements in the SHO1 branch; and a mutant of Hkr1 exhibits an altered kinetics of osmostress response.

An osmosensor detects either changes in extracellular water activity (direct osmosensing) or the resulting changes in the physical properties of cell structure (indirect osmosensing) (Wood, 1999). Although if and how Hkr1/Msb2 sense osmotic stress remains to be elucidated, our data would suggest that these molecules might directly monitor osmotic changes. The mucin-like STR domain is highly glycosylated, as has been demonstrated previously for Msb2 (Cullen *et al*, 2004). Organic polymer gels are highly sensitive to the solvent properties (Tanaka *et al*, 1980). Thus, it might be possible that a high osmolarity condition causes a significant volume change in the STR domain, thereby exposing the essential HMH domain and/or TM domains. It is also possible that Hkr1 and Msb2, individually, interact with another membrane protein forming an even larger osmosensing complex. This might explain why Hkr1-Δ(50–830), which has only one-fourth of the wild-type STR domain, is still capable of osmosensing (Figure 3G). In any case, the suggested model is consistent with a previous observation that activation of the SHO1 branch is independent of turgor changes (Reiser *et al*, 2003). Because turgor pressure requires the abutment of the plasma membrane and the cell wall, the turgor-based osmosensing mechanism employed by Sln1 is available only to walled cells, such as yeast, plant, and bacteria. In contrast, an oligosaccharide gel-based mechanism could be independent of the presence or absence of the cell wall, and thus is potentially available to animal cells. In this sense, Hkr1/Msb2 might offer a new paradigm of osmosensing utilized by higher mammalian cells as well.

The second finding is that there are actually two different activation mechanisms of the SHO1 branch (modes 1 and 2). In mode 1, Hkr1 (or Msb2) and Sho1, through their TM domains, interact with each other to generate an intracellular signal. The cytoplasmic domain of Hkr1/Msb2 is dispensable for the mode 1 mechanism, suggesting that the cytoplasmic signal is generated by associated Sho1. This idea is supported by the properties of the constitutively active Sho1-P120L mutant, which can activate the HOG pathway in the absence of the putative osmosensors Hkr1 and Msb2. The constitutively active Sho1-P120L might mimic a conformational change that is induced by activated Hkr1 or Msb2.

The mode 2 mechanism is less clearly understood at the moment. It is independent of the Sho1 TM domains and Hkr1, but instead the Msb2 cytoplasmic region is essential, suggesting that Msb2 itself, or an unidentified binding protein different from Sho1, generates a cytoplasmic signal. Unlike in mode 1, deletion of the STR region from Msb2 does not constitutively activate mode 2. This observation hints that there might be another membrane protein that is necessary for osmosensing and/or activation of Msb2 in mode 2. Thus, there are still many unknown factors in mode 2. Nonetheless, the two-mode model offers quite a robust framework to explain previous observations. For example, *Myr-SHO1* could complement *sho1*Δ, because mode 2 is functional, and *msb2*Δ mutants are not osmosensitive because mode 1 is functional.

The third finding is that Sho1 has at least two separable functions. One is that of receiving an osmostress signal from Hkr1 and Msb2 and converting it to an intracellular signal. The other is the previously described adaptor function of assembling Pbs2, Ste50, and Ste11 together through its cytoplasmic domain (Tatebayashi *et al*, 2006). The Myr-Sho1 construct is defective in signal transmission function, but it is fully capable of adaptor function. In contrast, the Sho1-W338F mutant is defective in the second (adaptor) function, but it can still generate a cytoplasmic signal (Figure 7B). The extracellular loops and TM domains of Sho1 are involved in the first function only, but the Sho1 cytoplasmic domain takes part in both functions. This will explain the previously puzzling finding that crosstalk activation of the mating pathways by osmostress requires the Sho1 cytoplasmic domain, but not its Pbs2-binding capacity (Marles *et al*, 2004).

In conclusion, this study has revealed a complex interplay among Hkr1, Msb2, and Sho1, in osmostress responses. The proposed model can explain the previous observations and serves as a basis for an integrated regulatory mechanism of the HOG and the mating/FG pathways.

Materials and methods

Yeast strains

The yeast strains used are listed in Supplementary Table I.

Media and buffers

Buffer D contains 50 mM Tris–HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol, 1% digitonin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 150 mM NaCl. Buffer Z contains 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, adjusted to pH 7.0. Other yeast media, buffers, and standard genetic procedures are as described previously (Rose *et al*, 1990; Tatebayashi *et al*, 2003).

Plasmids

MSB2 gene clones were gifts from P Cullen (SUNY at Buffalo) and J Pringle (UNC). The vector for Venus Fluorescent Protein (VeFP), pBS7, was obtained from the University of Washington Yeast Resource Center. VeFP is a more-efficiently maturing variant of yellow fluorescent protein (YFP) (Nagai *et al*, 2002). A mutation that is known to prevent dimerization of GFP, namely A206K (Zacharias *et al*, 2002), was introduced into VeFP to minimize its dimerization.

Isolation of constitutively active SHO1 mutants

Screening of constitutively active *SHO1* mutants was as described previously (Tatebayashi *et al*, 2006). Some *SHO1* mutants induced the *8xCRE-lacZ* reporter only in the presence of constitutively active Ste11-Q301P as previously reported, whereas others, such as *SHO1-P120L*, could induce the reporter in the *STE11* wild-type cells.

Isolation of Sho1 mutants defective in the mode 1 activation mechanism

A library of randomly mutagenized *SHO1* was placed into KT053 (*ssk2/22*Δ *sho1*Δ *msb2*Δ). Osmosensitive mutants were screened by replica-plating, and were further screened for those that became osmoresistant when mated with an $MSB2^{+}$ tester strain (*ssk2/22*Δ *sho1*Δ). In addition, each of the amino-acid residues Ser-61, Phe-62, and Pro-63 in the Sho1 extracellular loop 1 was changed to several different amino acids by site-directed mutagenesis. These *SHO1* mutant plasmids were re-introduced into QG153 (*ssk2/22*Δ *sho1*Δ) and KT053 carrying the *8xCRE-lacZ* reporter gene. Following osmotic stress, the levels of reporter expression in the two host cells was compared.

Reporter assays

The *8xCRE-lacZ* reporter assay has been described previously (Tatebayashi *et al*, 2006).

Fluorescence microscopy

Fluorescence microscopic images of exponentially growing cells were captured using a Nikon TE2000-E fluorescent microscope equipped with Photometrics Cool SNAP HQ CCD camera, as described previously (Tatebayashi *et al*, 2006). Confocal images were obtained using a Leica TCS-SP2-AOBS laser scanning microscope with a HCX PL APO lens (100×1.40 NA).

In vivo binding assay

Exponentially growing cells in CARaf were adjusted to 2% galactose and cultured for an additional 3 h. Cell extracts were prepared in buffer D using glass beads, essentially as described previously (Tatebayashi *et al*, 2003). A 750 μg aliquot of protein extract was incubated with 50 μl of glutathione-Sepharose beads for 2 h at 4°C. Beads were washed three times in buffer D, resuspended in SDS-loading buffer, incubated for 5 min at 37°C, and separated by SDS–PAGE. Immunoblots were probed with either the 12CA5 anti-HA antibody (Roche) or the B-14 anti-GST antibody (Santa Cruz), and detected by the ECL reagent (GE Healthcare). Images were digitally captured by LAS-1000 Plus (Fujifilm) equipped with a CCD camera.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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